

PRE-CLINICAL RESEARCH

Cannabidiol Attenuates Cardiac Dysfunction, Oxidative Stress, Fibrosis, and Inflammatory and Cell Death Signaling Pathways in Diabetic Cardiomyopathy

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- Objectives** In this study, we have investigated the effects of cannabidiol (CBD) on myocardial dysfunction, inflammation, oxidative/nitrative stress, cell death, and interrelated signaling pathways, using a mouse model of type I diabetic cardiomyopathy and primary human cardiomyocytes exposed to high glucose.
- Background** Cannabidiol, the most abundant nonpsychoactive constituent of *Cannabis sativa* (marijuana) plant, exerts anti-inflammatory effects in various disease models and alleviates pain and spasticity associated with multiple sclerosis in humans.
- Methods** Left ventricular function was measured by the pressure-volume system. Oxidative stress, cell death, and fibrosis markers were evaluated by molecular biology/biochemical techniques, electron spin resonance spectroscopy, and flow cytometry.
- Results** Diabetic cardiomyopathy was characterized by declined diastolic and systolic myocardial performance associated with increased oxidative-nitrative stress, nuclear factor- κ B and mitogen-activated protein kinase (c-Jun N-terminal kinase, p-38, p38 α) activation, enhanced expression of adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1), tumor necrosis factor- α , markers of fibrosis (transforming growth factor- β , connective tissue growth factor, fibronectin, collagen-1, matrix metalloproteinase-2 and -9), enhanced cell death (caspase 3/7 and poly[adenosine diphosphate-ribose] polymerase activity, chromatin fragmentation, and terminal deoxynucleotidyl transferase dUTP nick end labeling), and diminished Akt phosphorylation. Remarkably, CBD attenuated myocardial dysfunction, cardiac fibrosis, oxidative/nitrative stress, inflammation, cell death, and interrelated signaling pathways. Furthermore, CBD also attenuated the high glucose-induced increased reactive oxygen species generation, nuclear factor- κ B activation, and cell death in primary human cardiomyocytes.
- Conclusions** Collectively, these results coupled with the excellent safety and tolerability profile of CBD in humans, strongly suggest that it may have great therapeutic potential in the treatment of diabetic complications, and perhaps other cardiovascular disorders, by attenuating oxidative/nitrative stress, inflammation, cell death and fibrosis. (J Am Coll Cardiol 2010;56:2115–25) © 2010 by the American College of Cardiology Foundation

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Abbreviations and Acronyms

- ADP** = adenosine diphosphate
- CBD** = cannabidiol
- HCM** = human cardiomyocytes
- HG** = high glucose
- HNE** = hydroxynonenal
- ICAM** = intercellular adhesion molecule
- IκB-α** = inhibitor of nuclear transcription factor nuclear factor-κB
- INOS** = inducible nitric oxide synthase
- JNK** = c-Jun N-terminal kinase
- MAPK** = mitogen-activated protein kinase
- MMP** = matrix metalloproteinase
- NADPH** = nicotinamide adenine dinucleotide phosphate
- NF-κB** = nuclear factor kappa B
- NT** = nitrotyrosine
- PARP** = poly(ADP-ribose) polymerase
- ROS** = reactive oxygen species
- SOD** = superoxide dismutase
- THC** = delta 9-tetrahydrocannabinol
- TNF** = tumor necrosis factor
- TUNEL** = terminal deoxynucleotidyl transferase dUTP nick end labeling
- VCAM** = vascular cell adhesion molecule

Cardiovascular complications are the leading cause of morbidity and mortality in diabetic patients. Diabetic cardiomyopathy characterized by myocardial left ventricular dysfunction (both diastolic and later systolic), independent of atherosclerosis and coronary artery disease, has been well documented in both humans and animals (1-3). The mechanism of diabetic cardiac dysfunction is complex and involves increased oxidative/nitrative stress (4-7), activation of various downstream transcription factors, pro-inflammatory and cell death pathways such as nuclear factor (NF)-κB (8,9), poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) (10), and mitogen-activated protein kinase (MAPK) (11,12), inactivation of pro-survival pathways such as Akt (13), eventually culminating in cell death (14) and changes in the composition of extracellular matrix with enhanced cardiac fibrosis and increased inflammation (15).

Various components of the Cannabis sativa (marijuana) plant, termed cannabinoids (e.g., the most characterized active ingredient, the delta 9-tetrahydrocannabinol [THC]), exert potent analgesic effects through the activation of classic CB₁ receptors located in the central nervous system and anti-inflammatory properties through the activation of CB₂ cannabinoid receptors on immune cells (16). However, the major limitation of the therapeutic utility of

THC is the development of centrally mediated CB₁-dependent psychoactive effects (16). Furthermore, the CB₁ receptor activation in the cardiovascular system by endocannabinoids may also contribute to the pathophysiology of multiple cardiovascular diseases, including heart failure and atherosclerosis (17). In contrast to THC, cannabidiol (CBD), the most abundant cannabinoid of Cannabis sativa, which has been approved for the treatment of inflammation, pain, and spasticity associated with multiple sclerosis in humans since 2005 in Canada (18), does not bind to these receptors (19); therefore, it is devoid of psychoactive properties and has no potential to cause adverse cardiac toxicity (20). Importantly, CBD is well

tolerated without side effects when chronically administered to humans (21,22).

A previous study has demonstrated cardiac protection by CBD in myocardial ischemic reperfusion injury (23); therefore, we have investigated the potential protective effects of CBD in diabetic hearts and in primary human cardiomyocytes exposed to high glucose. Our findings underscore the potential of CBD for the prevention/treatment of diabetic complications.

Methods

Animals and treatment. All the animal protocols conformed to the National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee of National Institute on Alcohol Abuse and Alcoholism (NIAAA)-NIH. Diabetes mellitus was induced in male C57/BL6J mice 8 to 12 weeks old, weighing 23 to 25 g (Jackson Laboratories, Bar Harbor, Maine) by intraperitoneal injection of streptozotocin (Sigma, St. Louis, Missouri) at the dose of 50 mg/kg dissolved in 100 mM citrate buffer pH 4.5 for 5 consecutive days. After 1 week, blood glucose levels were measured using Ascensia Coutour Glucometer (Bayer HealthCare, Tarrytown, New York) by mandibular vein puncture blood sampling. Mice that had blood sugar values >250 mg/dl were used for the study. In the first set of experiments 1-week diabetic mice were treated with CBD (1, 10, or 20 mg/kg intraperitoneally) or vehicle for 11 weeks (Online Fig. 1). In another set of experiments, 8-week diabetic mice were treated with CBD or vehicle for 4 weeks (Online Fig. 2). The CBD was isolated as described earlier (24). The corresponding control groups were treated with either vehicle or CBD alone for the same duration. All the animals were provided with food and water ad libitum.

Hemodynamic measurements in mice. Left ventricular performance was measured in mice anesthetized with 2% isoflurane as previously described (25,26).

Determination of superoxide dismutase activity, malondialdehyde, reduced glutathione, oxidized glutathione, 4-HNE, and protein carbonyl content. The superoxide dismutase (SOD) activities, and reduced glutathione and oxidized glutathione, malondialdehyde, 4-HNE, and protein carbonyl levels in the myocardial tissues were determined as described in the Online Appendix.

Determination of myocardial reactive oxygen species (ROS) by electron paramagnetic resonance spectrometer is described in the Online Appendix.

Reverse transcription and real-time polymerase chain reaction. Preparation of samples and reverse transcription and real-time polymerase chain reaction (PCR) experiments from heart tissues and the primers are described in the Online Appendix and Online Table 1.

Determination of PARP, caspase 3/7 activities, chromatin fragmentation, TUNEL, and 3-NT content. The PARP and caspase 3/7 activities, chromatin fragmentation, terminal deoxynucleotidyl transferase dUTP nick end labeling

(TUNEL), and 3-nitrotyrosine (3-NT) content in the heart homogenates and/or human cardiomyocyte extracts are described in the Online Appendix.

Western immunoblot analysis. Sample preparations, Western immunoblot analysis, and sources of antibodies are described in the Online Appendix.

Immunohistochemistry. The immunohistochemistry/staining from frozen or formalin-fixed myocardial tissues (nitrotyrosine, TUNEL, Sirius red) is described in the Online Appendix.

Cell culture studies. Human cardiomyocytes (HCM) along with the culture medium were purchased from ScienCell Research Laboratories (Carlsbad, California) and were maintained and treated as described in the Online Appendix.

Simultaneous determination of cytosolic and mitochondrial ROS generation and apoptosis by flow cytometry. Mitochondrial superoxide/ROS generation and cell death were

determined as described (27) and are detailed in the Online Appendix.

Statistical analysis. Results are expressed as mean ± SEM. Statistical comparisons were made by 1-way analysis of variance followed by Newman-Keuls post-hoc analysis using GraphPad Prism 5 software (San Diego, California). When heterogeneity of variance was present, analysis of variance was performed after logarithmic transformation of the data. Probability values of $p < 0.05$ were considered significant.

Results

Blood glucose levels, pancreas insulin content, and body weights. Diabetic animals exhibited increased blood glucose levels (Online Figs. 1A and 2A) with the decrease in the body weight (Online Fig. 1D). Diabetic animals also had increased glycosylated hemoglobin (HbA_{1c}) levels

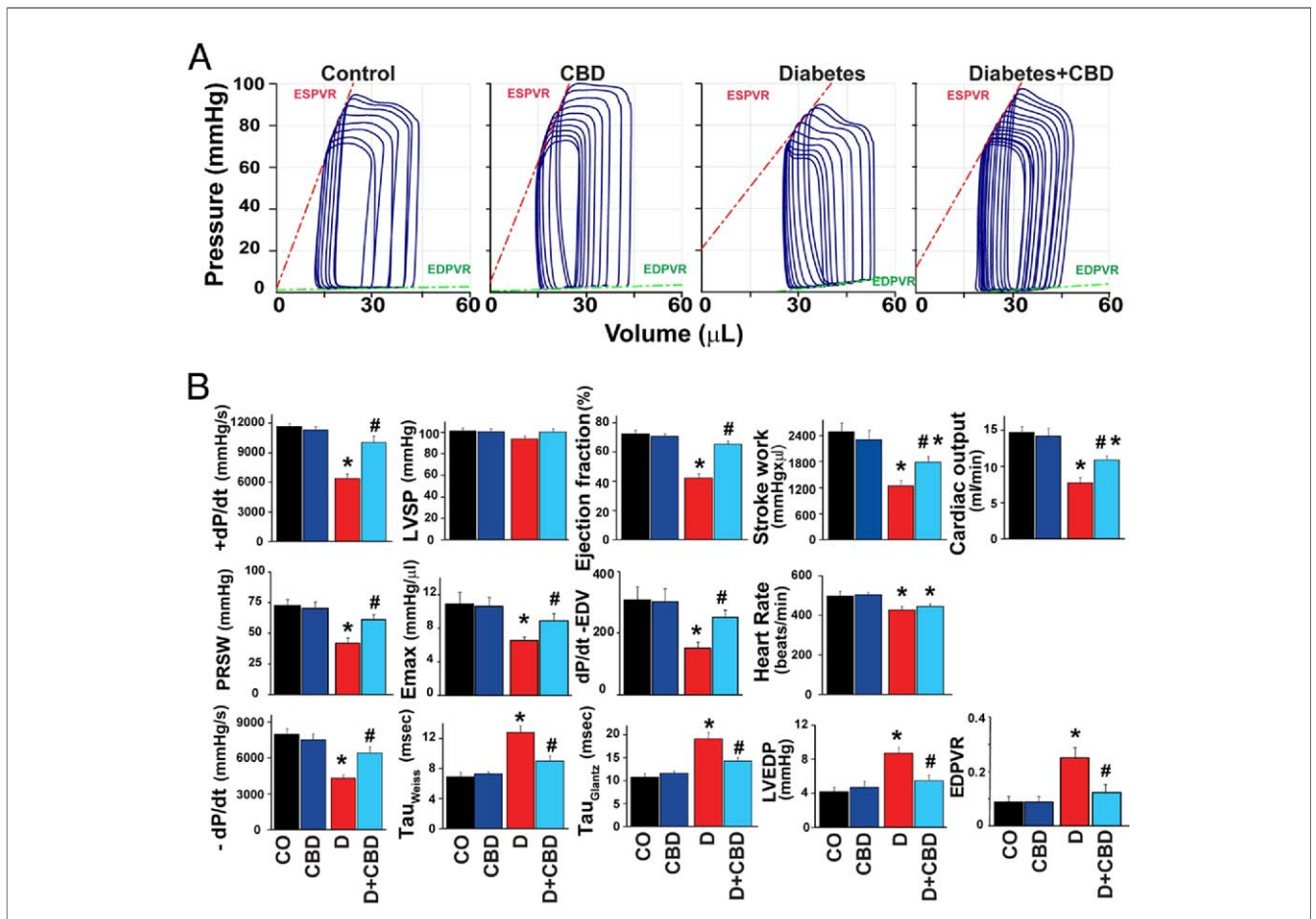


Figure 1 Cannabidiol Attenuates Diabetes-Induced Left Ventricular Dysfunction

(A) Representative pressure-volume (P-V) loops at different preloads after inferior vena cava occlusion, showing differences in the end-systolic P-V relations (ESPVR) and end-diastolic P-V relations (EDPVR) in control (Co) and diabetic mice treated with vehicle or cannabidiol (CBD). The shift of P-V loops right and changed slope of ESPVR and EDPVR in diabetic mice indicates decreased systolic and diastolic functions, which were less pronounced in diabetic mice treated with CBD (20 mg/kg daily) for 11 weeks. (B) Twelve weeks of diabetes was associated with decrease in left ventricular systolic pressure (LVSP), maximum first derivative of ventricular pressure with respect to time (+dP/dt), stroke work, ejection fraction, cardiac output, and load-independent indexes of contractility (pre-load–recruitable stroke work [PRSW], dP/dt–end-diastolic volume relation [dP/dt-EDV], and end-systolic pressure-volume relation [E_{max}], respectively), and an increase in left ventricular end-diastolic pressure (LVEDP) and prolongation of relaxation time constants (τ Weiss and Glantz), which were largely attenuated by CBD treatment (20 mg/kg daily intraperitoneally) for 11 weeks. Results are mean ± SEM of 8 to 11 per group. * $p < 0.05$ versus vehicle control/CBD alone; # $p < 0.05$ versus diabetes (D).

with concomitant decline in the pancreas insulin content (Online Fig. 1B and 2B). The CBD or vehicle treatment (1, 10, or 20 mg/kg intraperitoneally) for 11 or 4 weeks did not significantly alter the body weight, blood glucose level, or pancreas insulin content in either control or diabetic animals (Online Figs. 1 and 2).

CBD treatment attenuates diabetes-induced hemodynamic alterations. Twelve weeks of established diabetes was associated with impaired diastolic and systolic left ventricular function, which was largely attenuated by the treatment with CBD for 11 weeks (starting 1 week after the establishment of diabetes) (Fig. 1). The CBD treatment also improved the

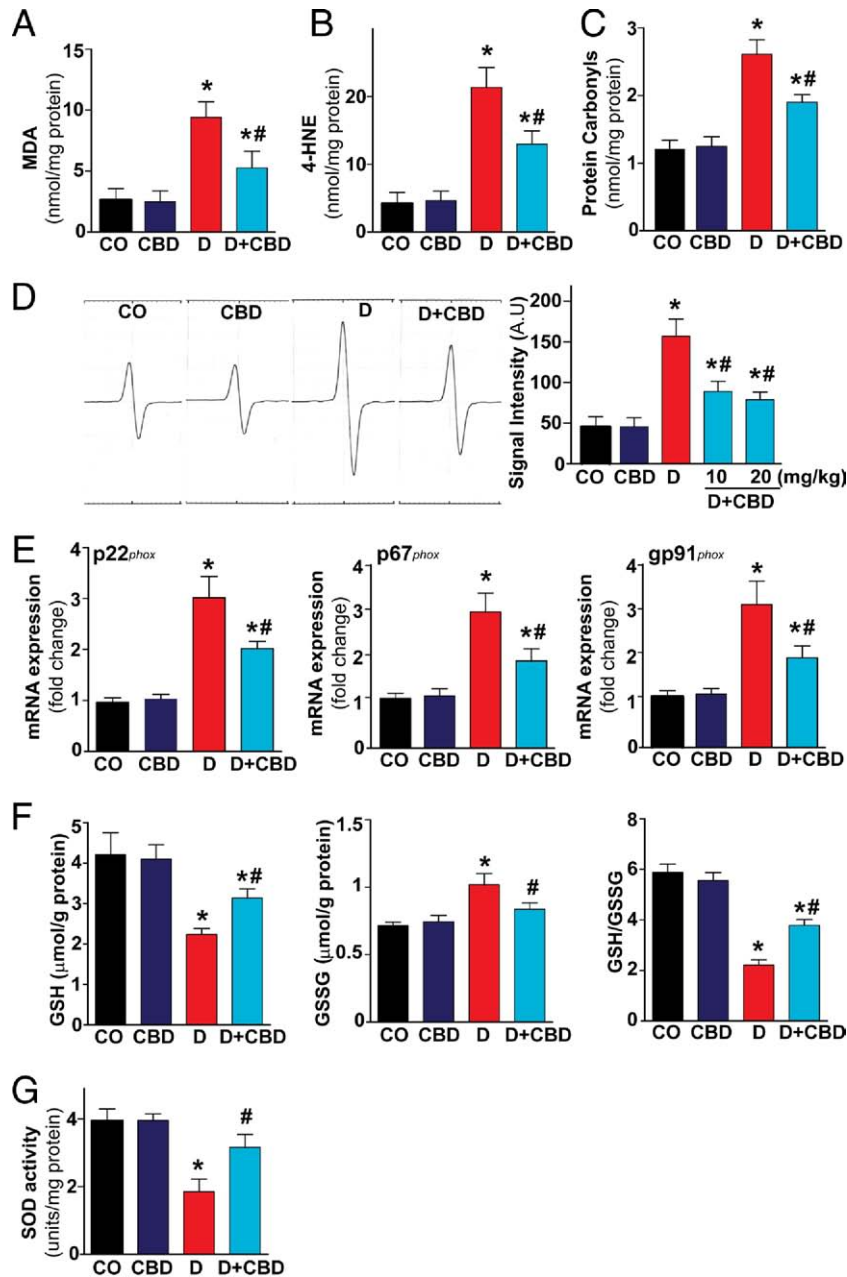


Figure 2 CBD Attenuates Diabetes-Induced Myocardial Oxidative Stress

Oxidative stress in the myocardial tissues were determined by measuring (A) malondialdehyde (MDA), (B) 4-HNE, (C) protein carbonyls content, and (D) reactive oxygen species (ROS) levels by electron paramagnetic resonance spectrometer, as described in the Methods section, and the (E) nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits messenger ribonucleic acid (mRNA) expression by real-time reverse transcriptase-polymerase chain reaction, (F) endogenous antioxidants (reduced glutathione [GSH] and oxidized glutathione [GSSG]) content, and (G) superoxide dismutase (SOD) activity. *p < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #p < 0.05 versus diabetes (D), n = 6 to 9 per group.

diabetes-induced myocardial dysfunction when it was given for 4 weeks in 8-week diabetic mice (Online Fig. 3).

CBD treatment attenuates diabetes-induced myocardial oxidative stress. There was increased accumulation of lipid peroxides (Figs. 2A and 2B), protein carbonyls (Fig. 2C), ROS generation (Fig. 2D), expression of messenger ribonucleic acid of various ROS-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (p22^{phox}, p67^{phox}, gp91^{phox}) (Fig. 2E) with concordant decrease of reduced/oxidized glutathione ratio (Fig. 2F) and attenuated activity of the superoxide-eliminating enzyme, the SOD (Fig. 2G), in hearts of diabetic mice. These changes were attenuated when mice were treated with CBD for 11 weeks during the course of the diabetes (Figs. 2A to 2G).

CBD treatment attenuates diabetes-induced myocardial nuclear factor- κ B activation and inflammation. As shown in Figure 3A, there was a marked inhibitor of nuclear transcription factor NF- κ B (I κ B- α) degradation in the cytosol of diabetic hearts, with increased phos-

phorylation of I κ B- α leading to release of active p65 NF- κ B, which subsequently translocates to the nucleus to induce the inflammatory and apoptotic gene expressions (Fig. 3B). Gel shift assay also confirmed the NF- κ B activation in diabetic hearts (Fig. 3C). The CBD treatment of diabetic mice inhibited the I κ B- α and subsequent p65NF- κ B nuclear translocation (Figs. 3A to 3C). The CBD treatment also inhibited the NF- κ B-dependent mRNA and/or protein expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Figs. 3D and 3F) and pro-inflammatory cytokine tumor necrosis factor (TNF)- α (Figs. 3E and 3G), respectively, in the diabetic myocardial tissues.

CBD treatments attenuates diabetes-induced nitritive stress. There was significant increase in inducible nitric oxide synthase (iNOS) expression (Fig. 4A) and 3-NT accumulation (Figs. 4B to 4E) in hearts of diabetic mice compared to vehicle or CBD alone treated mice. The CBD treatment attenuated the diabetes-induced iNOS

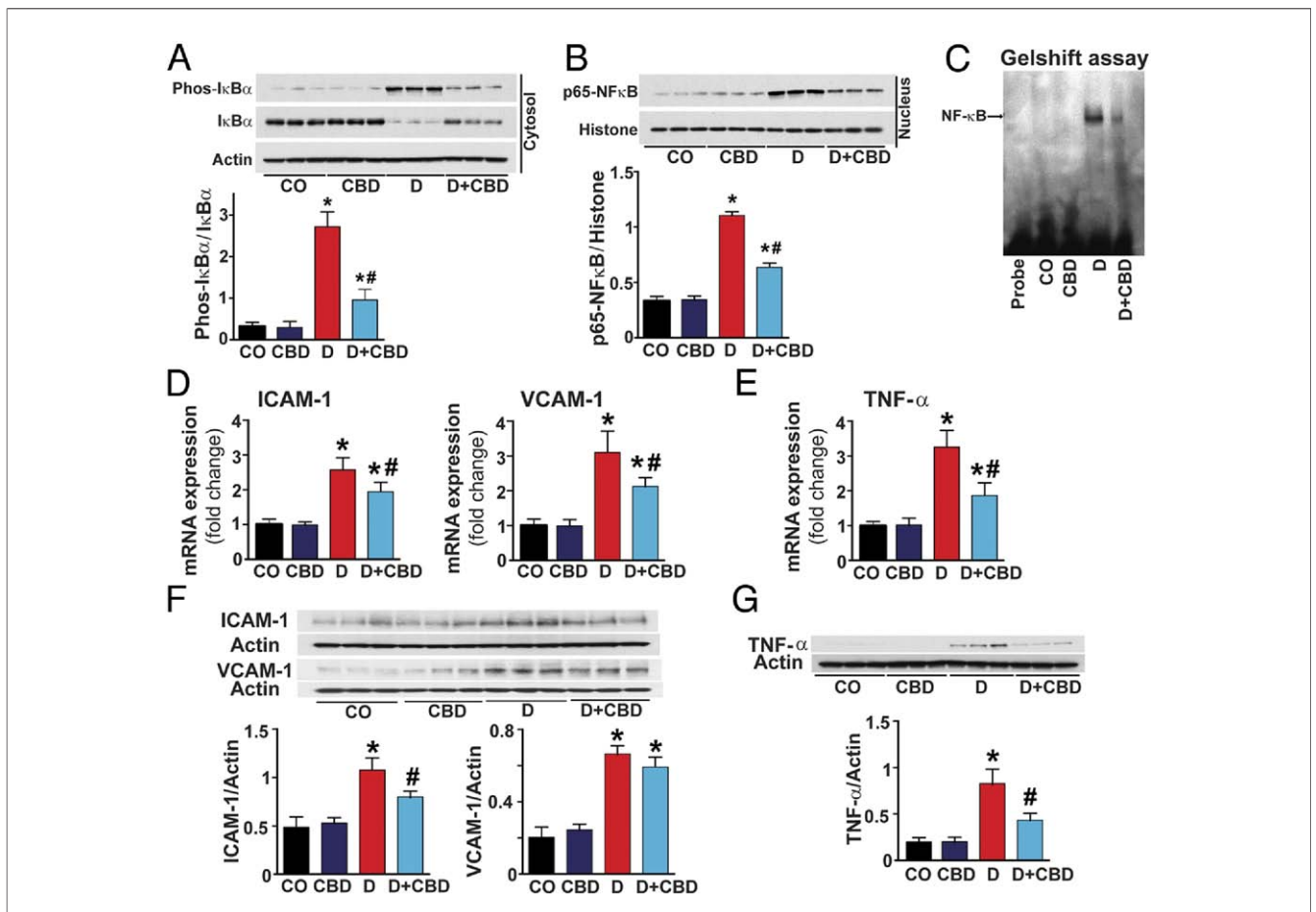


Figure 3 CBD Attenuates Diabetes-Induced Myocardial NF- κ B Activation

(A) Western blot analysis demonstrates inhibitor of nuclear transcription factor NF- κ B (I κ B- α) expression and its phosphorylation in the cytosolic fraction and (B) the nuclear translocation of p65 nuclear factor (NF)- κ B in the nuclear fraction of the heart tissue homogenates. (C) The gel shift assay demonstrates NF- κ B activation. (D) The messenger ribonucleic acid (mRNA) expression of ICAM (intercellular adhesion molecule)-1 and VCAM (vascular cell adhesion molecule)-1. (E) Tumor necrosis factor (TNF)- α in the respective groups, as indicated. (F) Western blot analysis for the protein expression of ICAM-1/VCAM-1, and (G) TNF- α protein in the myocardial tissues. * $p < 0.05$ versus vehicle control (Co) and cannabidiol (CBD) alone; # $p < 0.05$ versus diabetes (D), $n = 6$ to 9 per group.

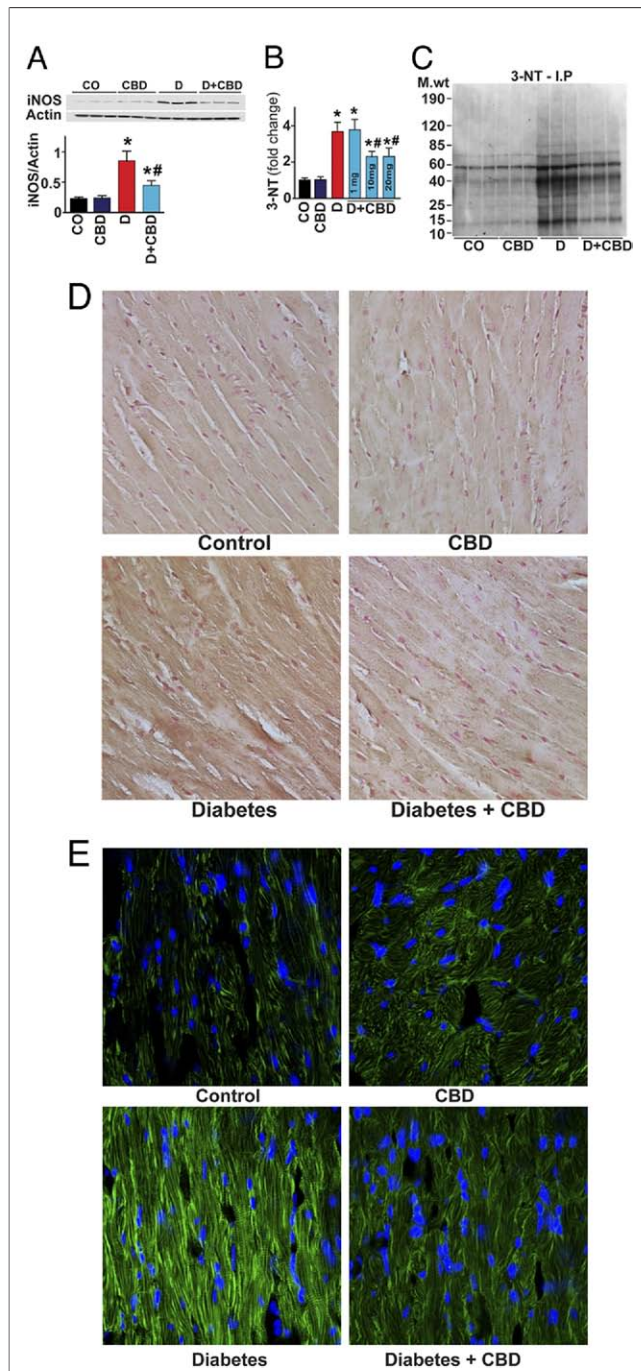


Figure 4 CBD Inhibits Diabetes-Induced Myocardial, iNOS Expression, and 3-NT Accumulation

(A) Expression of inducible nitric oxide synthase (iNOS) was determined by Western immunoblot in the heart tissues. (B) Levels of 3-nitrotyrosine (3-NT) in the heart samples were quantitatively determined by enzyme-linked immunosorbent assay with indicated cannabidiol (CBD) concentration (mg/kg body weight), respectively. (C) Representative gel indicates the nitrated proteins analyzed by immunoprecipitation (I.P) with 3-NT specific antibody. (D) Representative images for the histochemical staining for 3-NT accumulation in the formalin-fixed myocardial tissues (400× magnification). (E) Immunofluorescence staining for 3-NT from frozen sections as described in Methods (400× magnification). *p < 0.05 versus vehicle control (Co) and CBD alone; #p < 0.05 versus diabetes (D), n = 6 to 8 per group.

expression and 3-NT accumulation (marker of nitrative stress) (Figs. 4B to 4E).

CBD treatment attenuates diabetes-induced MAPK activation and apoptosis. There was marked increase in the p38MAPK (Fig. 5A) and c-Jun N-terminal kinase (JNK) (Fig. 5B) activation in the myocardial tissues of diabetic mice. In addition, there was marked activation p38 α MAPK (Fig. 5C) and slightly diminished p38 β MAPK (Fig. 5C) in the diabetic myocardium. There was also activation of MAPKAPK-2 in the diabetic heart (Fig. 5D). CBD treatment for 11 weeks significantly mitigated p38MAPK, JNK, p38 α MAPK, MAPKAPK-2 activation, while it was not effective in restoring the p38 β MAPK levels. In addition, Akt activation was also significantly hampered in the diabetic myocardium, which was attenuated with CBD treatment (Fig. 5E). In diabetic myocardium, there was marked increase in caspase 3 cleavage, caspase 3/7 activity (Figs. 6A and 6B), chromatin fragmentation, and PARP activity (Figs. 6C and 6D), and enhanced apoptosis (Figs. 6E and 7); all these changes in diabetes were attenuated by CBD treatment.

CBD treatment attenuates diabetes-associated myocardial fibrosis. Real-time reverse transcriptase-polymerase chain reaction analysis revealed significant increases in the pro-fibrotic gene expressions (Fig. 8A) and in collagen deposition (Fig. 8B) in diabetic hearts, and these were attenuated by CBD (Fig. 8).

CBD post-treatment after the establishment of diabetic cardiomyopathy attenuates diabetes-induced myocardial oxidative/nitrative stress, cell death, and fibrosis. Remarkably, CBD 20 (mg/kg) treatment also attenuated the diabetes-induced increased myocardial nitrative stress, cell death (Online Fig. 4) and fibrosis (Online Fig. 5) when it was given for 4 weeks in 8-week diabetic mice.

CBD treatment attenuates high glucose-induced cytosolic and mitochondrial ROS generation and 3-NT formation in HCM. High glucose (HG) treatment of HCM for 48 h markedly increased cytosolic (Online Fig. 6A) and mitochondrial (Online Fig. 6B) ROS/superoxide generation compared with cells treated with either D-glucose 5 mM, L-glucose 30 mM, or CBD (4 μ M) alone for the same duration. The CBD markedly attenuated the HG-induced increased ROS generation (Online Figs. 6A and 6B) and 3-NT accumulation in HCM (Online Fig. 6C).

CBD mitigates HG-induced NF- κ B activation and apoptosis in HCM. The HG treatment induced NF- κ B activation (Online Figs. 7A and 7B) and increased apoptosis and PARP-dependent cell death in cardiomyocytes (Online Figs. 8A and 8B); the antiapoptotic activity of CBD was mediated, at least in part, by its ability to modulate Akt activity (Online Fig. 8A).

Discussion

Accumulating evidence suggests that increased oxidative/nitrative stress coupled with activation of various down-

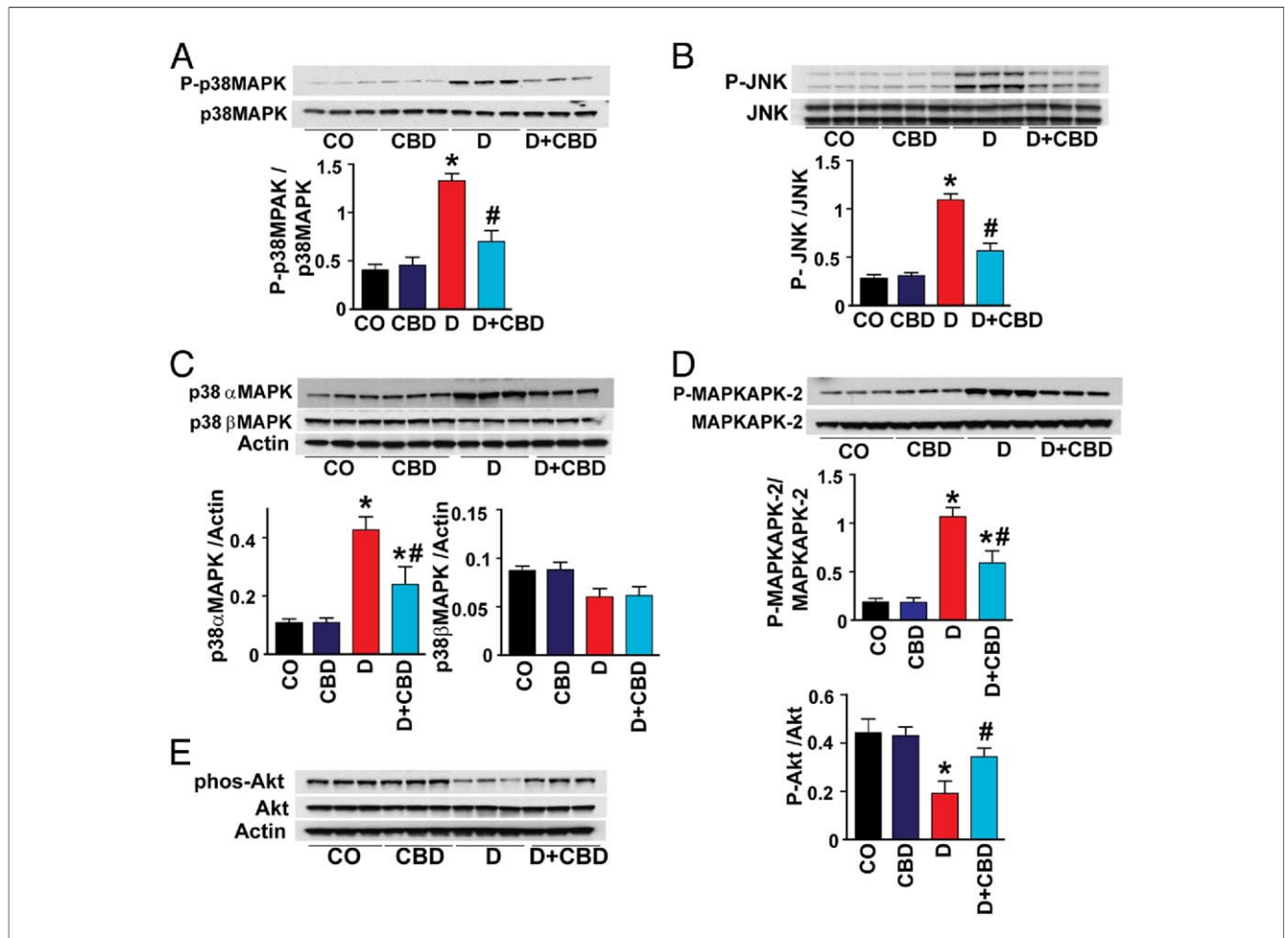


Figure 5 CBD Mitigates Diabetes-Induced Myocardial Activation of MAPKs and Augments Akt Activation

Western blot analysis shows the (A) p38 mitogen-activated protein kinase (MAPK), (B) c-Jun N-terminal kinase (JNK), (C) p38α/βMAPK, (D) MAPKAPK-2, and (E) Akt activation in the myocardial tissues. *p < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #p < 0.05 versus diabetes (D), n = 6 per group.

stream pro-inflammatory and cell death pathways play pivotal roles in the development of complex biochemical, mechanical, and structural alterations associated with diabetic cardiomyopathy (3,4,6,11,12,14,15). However, in spite of the accumulating knowledge obtained during the past decades, the treatment of diabetic cardiomyopathy still remains poor and largely symptomatic (1,2).

Cannabidiol, a nonpsychoactive component of marijuana, has been shown to exert anti-inflammatory and antioxidant effects both in vitro and in various preclinical models of neurodegeneration and inflammatory disorders, independent from classical CB₁ and CB₂ receptors (20). Furthermore, CBD has recently been reported to lower the incidence of diabetes among nonobese diabetic mice (28) and to preserve the blood-retinal barrier in experimental diabetes (29).

In the present study, we have evaluated the effects of CBD treatment (for 11 weeks administered after the destruction of pancreatic beta cells and development of frank type 1 diabetes mellitus, as well as in 8-week diabetic animals for 4 weeks) on myocardial dysfunction, inflamma-

tion, oxidative/nitrative stress, cell death, and interrelated signaling pathways, using a mouse model of type I diabetic cardiomyopathy or primary human cardiomyocytes exposed to HG. Because significant cardiac dysfunction in this model starts to develop from 4 weeks of established diabetes (4,10), with gradually increasing fibrosis thereafter (12,15) (peaking around 8 weeks of established diabetes), in the first treatment protocol (Online Fig. 1A.), we aimed to study if CBD treatment can prevent the development of characteristic alterations of type I diabetic cardiomyopathy; in the second treatment protocol (Online Fig. 2A), we sought to determine if it is able to reverse these changes once they have already developed.

Consistent with previous reports, diabetic cardiomyopathy was characterized by declined diastolic and systolic myocardial performance associated with enhanced myocardial expression of NADPH oxidase isoforms p22^{phox}, p67^{phox}, gp91^{phox}, attenuated antioxidant defense (decreased glutathione content and SOD activity) coupled with increased myocardial ROS generation and lipid peroxida-

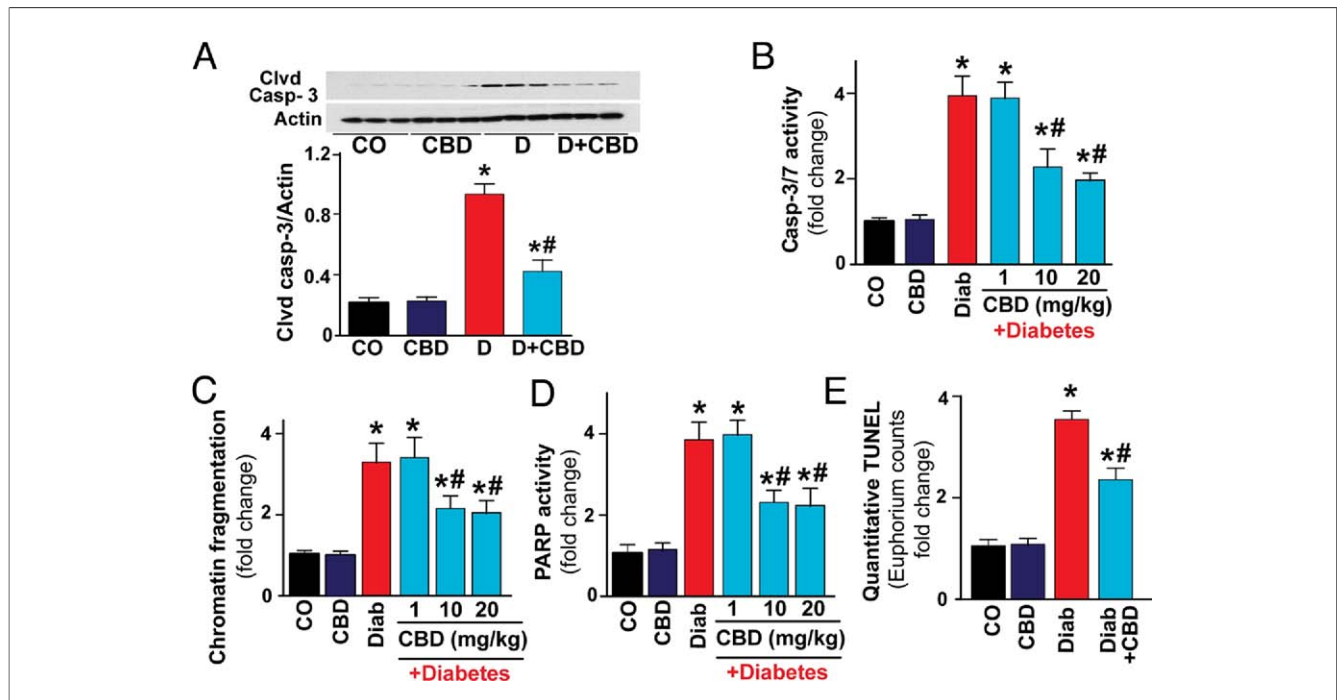


Figure 6 CBD Mitigates Diabetes-Induced Myocardial Apoptosis and Cell Death

(A) Western blot analysis for the cleaved (Clvd) caspase (Casp) 3 and (B) caspase 3/7 activity, (C) chromatin fragmentation, and (D) poly(ADP-ribose) polymerase (PARP) activation and (E) quantitative terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay were performed, as described in Methods. * $p < 0.05$ versus vehicle control (Co) and cannabidiol (CBD) alone; # $p < 0.05$ versus diabetes (D), $n = 6$ to 9 per group.

tion (4,6,10,12,15). The HG-induced ROS generation in addition to inducing lipid peroxidation may also initiate activation of various stress signaling pathways (e.g., jun

N-terminal kinase and p38MAPK). Our results are also in agreement with previous studies demonstrating enhanced activation of p38MAPK and its downstream effector

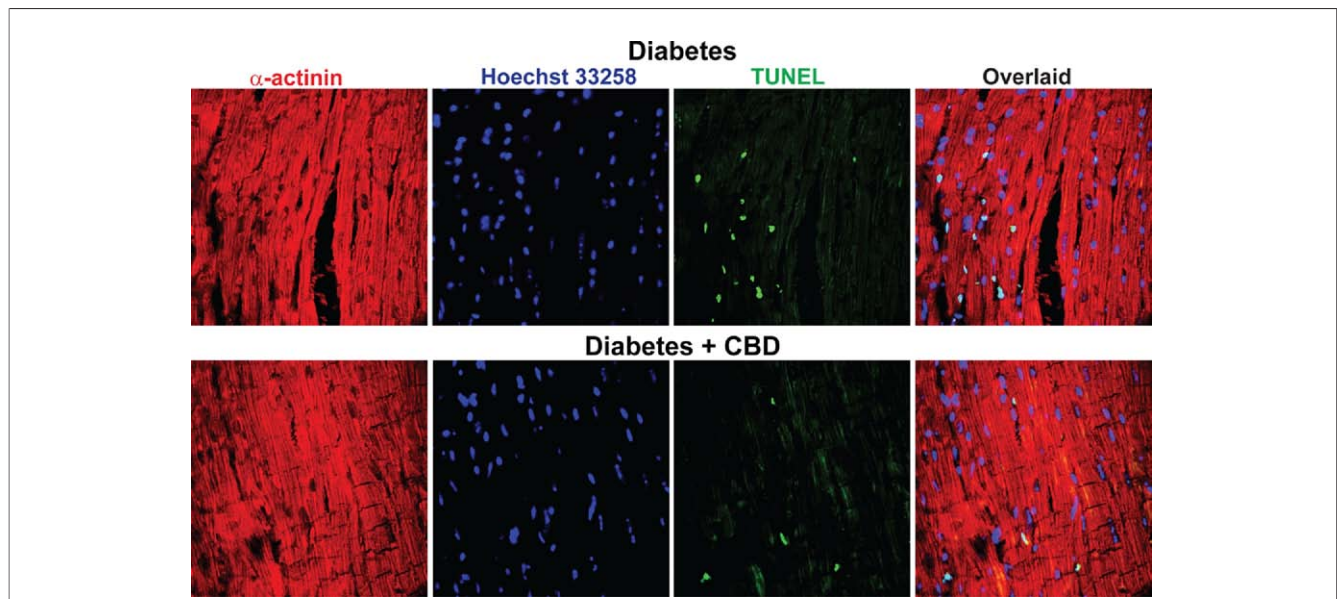


Figure 7 CBD Mitigates Apoptosis in the Diabetic Myocardium

Shown are the representative terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) images in the diabetic myocardium and mice that were treated with cannabidiol (CBD) for 11 weeks. For details, see Online Appendix Supplemental Methods.

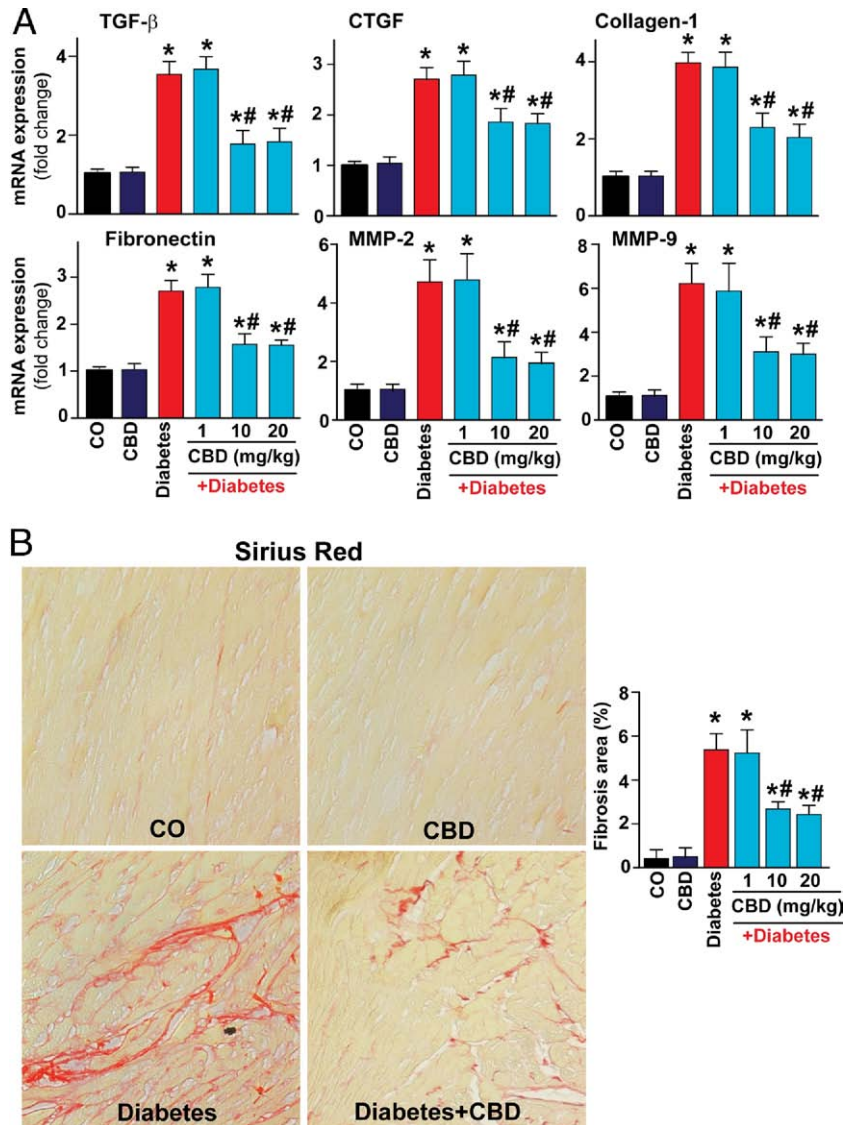


Figure 8 CBD Attenuates Diabetes-Induced Cardiac Fibrosis

(A) Messenger ribonucleic acid (mRNA) expression of the profibrotic genes in the myocardial tissues. **p* < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #*p* < 0.05 versus diabetes (D), *n* = 9 per group. (B) Sirius red staining indicating collagen deposition and implying the extent of cardiac fibrosis. Images shown are representative from 4 independent experiments. **p* < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #*p* < 0.05 versus diabetes (D), *n* = 4 to 6 per group. CTGF = connective tissue growth factor; MMP = matrix metalloproteinase; TGF = transforming growth factor.

(p38MAPKAPK-2) in diabetic cardiomyopathy models and demonstrating that pharmacological inhibition of p38MAPK signaling attenuates the expression of cardiac inflammatory markers, such as TNF- α , interleukin-1 β , and interleukin-6, and collagen content associated with diabetic cardiomyopathy (11,12). Likewise, with recent evidence supporting an emerging role of p38 α activation in diabetic cardiomyopathy (12), in addition to its already established role in mediating cell death during myocardial ischemic-reperfusion injury. The HG-induced ROS generation also impairs important pro-survival signaling pathways such as Akt in diabetic hearts (13), activates

pro-inflammatory and cell death pathways such as NF- κ B (8,9) and nuclear enzyme poly(ADP)-ribose polymerase 1 (10), which in turn regulate expression of important pro-inflammatory cytokines, cell adhesion molecules, and iNOS. The latter results in increased nitrosative/nitrative stress, which is also implicated in cardiovascular complications of diabetes (5). A recent study has also suggested that the NF- κ B activation may induce increased oxidative stress and contributes to mitochondrial and cardiac dysfunction in type II diabetes (9). Importantly, the oxidative-nitrative stress, stress signaling, and inflammatory pathways in diabetic cardiomyopathy are closely interrelated, even-

tually promoting the development of myocardial fibrosis (3,9,11,15,30).

Treatment with CBD (Supplemental Fig. 1) was able to attenuate the oxidative-nitrative stress (decreased the myocardial ROS generation and expression of p22^{phox}, p67^{phox}, gp91^{phox}, restored glutathione content and SOD activity, decreased 3-NT formation) and alterations of the pro-survival (Akt) and stress signaling (p38, p38 α , JNK) pathways in diabetic hearts. It also attenuated the NF- κ B activation, expression of iNOS, TNF α , and ICAM-1, cell death, and fibrosis in diabetic myocardium, and improved the associated characteristic functional alterations. Importantly, CBD treatment was able to attenuate/reverse (although to a lesser extent) some of the discussed diabetes-induced myocardial biochemical and functional changes after the establishment of diabetic cardiomyopathy with fibrosis (Online Figs. 2 to 5). The CBD treatment also attenuated the HG-induced increased reactive oxygen and nitrogen species generation, NF- κ B activation, and cell death in primary human cardiomyocytes (Online Figs. 6 to 8).

The discussed beneficial effects of CBD could be explained in part by its potent antioxidant properties, which was first suggested by the Nobel Prize winner Dr. Julius Axelrod (31). In the Axelrod study, CBD was more protective against glutamate-induced neurotoxicity than any of the well-known antioxidants (e.g., ascorbate or α -tocopherol), indicating additional cytoprotective effects of CBD beyond its potent antioxidant properties (31). Indeed, our recent results suggest that CBD may exert potent effects on key pro-inflammatory pathways such as NF- κ B and on pro-survival signaling such as Akt *in vivo*, which is most likely not related to its antioxidant effect. This is also supported by observations that CBD decreases inflammation in models in which conventional antioxidants are not very effective (e.g., in arthritis [20,32]), as well as by recent studies demonstrating that CBD is a potent inhibitor of bacterial lipopolysaccharide-activated NF- κ B proinflammatory pathway in microglia cells (33). These results are also in support of the emerging role of the inflammation in the development and progression of diabetic cardiomyopathy (9,11,15,30).

Collectively, our results strongly suggest that CBD may have tremendous therapeutic potential in the treatment of diabetic cardiovascular and other complications by attenuating diabetes-induced oxidative/nitrative stress, inflammation, cell death, and fibrotic pathways.

Acknowledgments

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Key Words: cannabinoids ■ diabetic complications ■ inflammation ■ oxidative stress.

 **APPENDIX**

For a detailed discussion of the Methods, supplemental references, table, and figures, please see the online version of this article.